Epidermal growth factor receptor and ligand family expression and activity in glioblastoma

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Abstract: Epidermal growth factor family of receptor tyrosine kinases (ERBB) family cell surface receptors, including epidermal growth factor receptor (EGFR/ERBB1), are phosphorylated upon binding by various EGF family ligands and signal via multiple kinase pathways. EGFR signaling is enhanced because of mutational activation of EGFR in almost half of glioblastomas, the most common malignant primary brain tumor. Therapeutic targeting of EGFR in glioblastoma has remained largely unsuccessful. Here, we profiled nine long-term (LTC) and five glioma-initiating (GIC) cell lines for expression and activation of ERBB family receptors and expression of their ligands. Receptors and ligands were abundantly expressed, with patterns overall similar to glioblastoma expression profiles in vivo as deposited in The Cancer Genome Atlas database. No differences between LTC and GIC emerged. Irrespective of ligand or receptor expression, neither an EGFR antibody, erbitux, nor an EGFR tyrosine kinase inhibitor, gefitinib, were particularly active against LTC or GIC at clinically relevant concentrations. Self-renewal capacity of GIC was severely compromised by epidermal growth factor (EGF) withdrawal, but rescued by transforming growth factor alpha (TGF-), although not by neuregulin-1 (NRG-1). Subcellular fractionation indicated high levels of nuclear phosphorylated EGFR in all LTC and GIC. In LN-229 cells, pERBB2 and pERBB3 were also detected in the nucleus. Nuclear pERBB2 was less sensitive, whereas pERBB3 was induced, in response to gefitinib. This study provides an extensive characterization of human glioma cell models, including stem-like models, with regard to ERBB receptor/ligand expression and signaling. Redundant signaling involving multiple ERBB family ligands and receptors may contribute to the challenges of developing more effective EGFR-targeted therapies for glioblastoma.

DOI: https://doi.org/10.1111/jnc.14538

Epidermal growth factor receptor and ligand family expression and activity in glioblastoma

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Running title: ERBB receptor and ligand family activity in glioblastoma

Keywords: glioma, EGF, ERBB, glioma-initiating cells

Abbrevations: protein kinase B, AKT; amphiregulin, AREG; ~~basic~~ fibroblast growth factor, ~~bFGF~~FGF; betacellulin, BTC; ~~Dulbecco~~dulbecco´s modified Eagle´s medium, DMEM; epidermal growth factor receptor, EGFR; epidermal growth factor family of receptor tyrosine kinases, ERBB: epiregulin, EREG; fetal calf serum, FCS; glioma-initiating cell, GIC; heparin-binding epidermal growth factor, HB-EGF; human epidermal growth factor receptor, HER; hypoxanthine-guanine phosphoribosyltransferase 1, HPRT1; isocitrate dehydrogenase, IDH; immunohistochemistry, IHC; janus kinase/signal transducer and activator of transcription, JAK/STAT; long-term cell line, LTC; mitogen-activated protein kinase, MAPK; neuregulin, NRG; neurobasal medium, NB; phosphorylated EGFR, pEGFR; ~~receptor tyrosine~~ phosphoinositide 3-kinase, ~~RTK~~PI3K; radio-immunoprecipitation assay, RIPA; receptor tyrosine kinase, RTK; transforming growth factor, TGF; D9-tetrahydrocannabinol, THC; tyrosine kinase ~~inhibitors~~inhibitor, TKI~~.~~; The Cancer Genome Atlas; TCGA

Abstract

~~Epidermal~~

ERBB family cell surface receptors, including epidermal growth factor receptor (EGFR~~)~~ ~~-~~/ERBB1), are phosphorylated upon binding by various EGF family ligands and signal via multiple kinase pathways. EGFR signaling is enhanced due to mutational activation ~~or overexpression~~ of EGFR in ~~more than~~almost half of glioblastomas~~. Still, pharmacological~~, the most common malignant primary brain tumor. Therapeutic targeting of EGFR in glioblastoma has remained largely unsuccessful ~~in glioblastoma. .~~

Here we ~~explored EGFR pathway activation in a panel of~~profiled 9 long-term (LTC) and 5 glioma-initiating (GIC) cell lines~~. Since *EGFR* mRNA, EGFR protein and phosphorylated EGFR (pEGFR) did not correlate, we extended the analyses to determine mRNA and protein levels of the other EGFR family members, ERBB2,3, and 4, and mRNA~~ for expression ~~of the EGF family ligands, EGF, transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), betacellulin (BTC), and epiregulin (EREG).~~and activation of ERBB family receptors and expression of their ligands. Receptors and ligands were abundantly expressed ~~in the cell line panel~~, with patterns overall similar to ~~glioblastomas~~glioblastoma expression profiles *in vivo* ~~in the~~ as deposited in The Cancer Genome Atlas (TCGA) database. No differences between LTC and GIC emerged. Irrespective of ligand or receptor expression, neither ~~a~~an EGFR antibody, erbitux, nor an EGFR tyrosin kinase ~~receptor~~ inhibitor (TKI), gefitinib, ~~nor an EGFR antibody, erbitux,~~ were particularly active against LTC or GIC at clinically relevant concentrations. ~~The self~~Self-renewal capacity of GIC was severely compromised by EGF withdrawal~~. This effect was~~ , but rescued by ~~TGF-α, but~~ transforming growth factor alpha (TGF- α), although not by neuregulin-1 (~~NRG1).~~NRG-1). Subcellular fractionation indicated high levels of nuclear phosphorylated EGFR in all LTC and GIC. In LN-229 cells, pERBB2 and pERBB3 were also detected in the nucleus. Nuclear pERBB2 was less sensitive, whereas pERBB3 was induced, in response to gefitinib.

This study provides an extensive characterization of human glioma cell models, including stem-like models, with regard to ERBB receptor/ligand expression and signaling. Redundant signaling involving multiple ERBB family ligands and receptors

may contribute to the challenges of developing more ~~efficacious~~effective EGFR-targeted therapies for glioblastoma.

Introduction

The ERBB protein family consists of 4 members~~:~~, ERBB1, ERBB2, ERBB3 and ERBB4~~. *EGFR (ERBB1)*~~, which are activated upon ligand binding to the extracellular ligand binding domain, triggering receptor homo- or heterodimerization and phosphorylation of certain cytoplasmic tyrosine residues (Hynes & Lane 2005, Yarden & Sliwkowski 2001). Tyrosine autophosphorylation leads to the recruitment and activation of multiple targets and pathways, e.g., the mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (Olayioye *et al.* 2000, Hynes *et al.* 2001, Yarden & Sliwkowski 2001). ERBB family proteins are activated by a group of related ligands: epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and amphiregulin (AR) bind specifically to epidermal growth factor receptor (EGFR/ERBB1), whereas heparin-binding epidermal growth factor (HB-EGF), betacellulin (BTC) and epiregulin (EREG) bind to ERBB1 and ERBB4. The neuregulins (NRG1, 2, 3, 4) bind either to ERBB3 and ERBB4 (NRG1,2) or to ERBB4 specifically (NRG3,4). Since none of the ligands can bind to ERBB2 alone and since ERBB3 has weak autophosphorylation properties, both receptors function mainly through heterodimerization with other ERBB receptors, preferentially with each other (Hynes et al. 2001, Yarden & Sliwkowski 2001, Graus-Porta *et al.* 1997, Steinkamp *et al.* 2014, Shi *et al.* 2010). *EGFR* is the most frequently amplified gene in glioblastoma (40%), resulting in increased ~~ERBB1~~EGFR mRNA and protein levels, and exhibits mutation, rearrangement, deletions and altered splicing, leading to the expression of different aberrant transcript variants (Cancer Genome Atlas Research 2008, Brennan *et al.* 2013). *EGFR* gene amplification is common in the classic or receptor tyrosine kinase (RTK) type 2 molecular subtype of isocitrate dehydrogenase (*IDH*)-wildtype glioblastoma (Sturm *et al.* 2012, Brennan et al. 2013). Half of *EGFR*-amplified glioblastomas carry a tumor-specific deletion variant (EGFRvIII) characterized by an in-frame deletion of exons 2-7, resulting in constitutive EGFR activation (Felsberg *et al.* 2017). Most studies of ERBB receptor family expression beyond EGFR in gliomas have used immunohistochemistry ~~(IHC)~~ techniques (Torp *et al.* 2007, Duhem-Tonnelle *et al.* 2010, Weller *et al.* 2014, Felsberg et al. 2017)~~. In the absence of gene amplification, increased immunoreactivity for ERBB proteins in glioblastoma~~

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~~may result from the receptor conformation status which depends on the activation level and modified dimerization profile rather than overexpression at the protein level alone (Duhem-Tonnelle et al. 2010).~~. ERBB1 and ERBB2 ~~receptors~~ exhibit highly variable expression profiles among glioblastoma samples, whereas ERBB3 and ERBB4 are expressed at lower levels than in control (non-neoplastic cerebral cortex) tissues. However, ERBB3 may be prominently expressed in CD133-positive putative tumor stem cells (Duhem-Tonnelle et al. 2010). Similarly, genes encoding the ERBB ligands *EGF*, ~~transforming growth factor (~~*TGF*~~)~~ α, ~~heparin-binding epidermal growth factor (~~*HB-EGF*~~), betacellulin (~~, *BTC*~~)~~ and ~~epiregulin (~~*EREG*~~)~~ were reported to exhibit highly heterogeneous expression profiles among glioblastoma samples and derived cell lines. ~~However, most neuregulin (~~*NRG1, 2, 3, 4*~~)~~ genes ~~are~~were expressed at low levels relative to cerebral cortex (Duhem-Tonnelle et al. 2010).

~~Exploiting EGFR as a therapeutic target in glioblastoma has remained challenging. Glioblastoma cell resistance to pharmacological EGFR inhibitors such as antibodies (cetuximab) or tyrosine kinase inhibitors (TKI) may be explained by compensatory activation of EGFR related family members (ERBB2, ERBB3), enabling persistent glioblastoma cell proliferation,~~

Exploiting EGFR as a therapeutic target in glioblastoma has remained challenging (Furnari *et al.* 2015). Glioblastoma cell resistance to pharmacological EGFR inhibitors including antibodies, such as cetuximab or tyrosine kinase inhibitors (TKI) such as gefitinib or erlotinib has been attributed to multiple reasons. The blood brain barrier may limit access to large molecules or hydrophilic molecules in tumor areas where the barrier is not disrupted (Lassman *et al.* 2005). Primary redundance of pathway activation, or compensatory pathway activation in response to interventions, may maintain down-stream signaling even when EGFR signaling is blocked (Hegi *et al.* 2011). This may involve EGFR-related family members such as ERBB2 or ERBB3 since dual inhibition of EGFR and ERBB2 with lapatinib significantly reduced glioblastoma cell proliferation compared to cetuximab (Clark *et al.* 2012)~~.~~. ERBB2 itself has also attracted interest as a target structure for natural killer cell-based immunological treatment approaches in various cancers, including glioblastoma (Nowakowska *et al.* 2018, Ahmed *et al.* 2017). In contrast to the antibodies, cetuximab and trastuzumab, selective siRNA-mediated gene silencing of *EGFR* or *ERBB2* in glioblastoma cells, reduced the growth rate *in vitro* by 40% and 65%, respectively. EGFR gene silencing did not change migration, however, silencing of

EGFR and ERBB2 reduced migration by 50% and induced radiosensitization in U251MG cells (Wichmann *et al.* 2015). T98G glioma cells incubated with antisense oligonucleotides to TGF-α exhibited growth inhibition in vitro (Rubenstein *et al.* 2001). Silencing of HB-EGF in U87MG cells, conditionally expressing either EGFRwt or EGFRvIII, attenuated EGFRvIII phosphorylation, inhibiting EGFRvIII-induced tumorigenicity, suggesting that an HB-EGF-EGFR/EGFRvIII loop regulates EGFRvIII activation (Li *et al.* 2014). Inhibition of *NRG1* expression by siRNA reduced the mRNA levels of L1, a cell adhesion molecule, responsible for migration of glioblastoma cells *in vitro* (Zhao & Schachner 2013). Silencing of ~~amphiregulin (~~AREG~~),~~ another ERBB ligand, reduced tumor growth by itself and rendered C6-4 glioma cell-derived tumors sensitive to D9-tetrahydrocannabinol (THC) treatment (Lorente *et al.* 2009). Neutralizing antibodies to ~~epiregulin (~~EREG~~)~~ decreased cell proliferation in U87MG cells *in vitro* (Auf *et al.* 2013). Altogether, these studies indicate that ERBB signaling beyond EGFR may determine the biological behavior of glioblastoma cells, notably in the setting of pharmacological interference with EGFR signaling.

Given the increasing interest in exploiting ERBB family receptors as targets for experimental therapy in brain tumors, which presently include vaccines (Weller *et al.* 2017b), chimeric antigen receptor T cells (Sampson *et al.* 2014) and antibody drug conjugates (Gan *et al.* 2017), the present study was conducted to provide a comprehensive characterization of ERBB family ligand and receptor expression in a large set of human glioma models, including glioma-initiating cell (GIC) models.

Materials and Methods

Reagents and cell lines

Gefitinib (Iressa®) is an EGFR selective ~~tyrosine kinase inhibitor~~TKI purchased from InvivoGen (tlrl-gef, San Diego, CA). Cetuximab (Erbitux®) was kindly provided by Merck (Darmstadt, Germany). Nine long-term glioblastoma cell lines (LTC) (LN-18 (RRID:CVCL_0392), LN-428 (RRID:CVCL_3959), D247 (RRID:CVCL_1153), LN-319 (RRID:CVCL_3958), A172 (RRID:CVCL_0131), U87MG (RRID:CVCL_0022), T98G (RRID:CVCL_0556), LN-308 (RRID:CVCL_0394), LN-229 (RRID:CVCL_0393)) (Weller *et al.* 1998) were maintained in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Five glioma-initiating cell lines (GIC) (T-325, T-269, ZH-161 (RRID:CVCL_JZ64), S-24, ZH-305 (RRID:CVCL_JZ65)) were isolated from surgically removed glioblastomas -(Lemke *et al.* 2014, Seystahl *et al.* 2015, Silginer *et al.* 2016) and cultured as spheres in neurobasal medium (NB) supplemented with 2% B27, 1% glutamine, EGF (20 ng/ml), and basic fibroblast growth factor (~~bFGF~~FGF, 20 ng/ml~~).~~) (Table S1). They have never been exposed to FCS. LTC and GIC are routinely authenticated at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures in Braunschweig, Germany, last in 2016. LN-319 and U87MG are part of our panel of standard cell lines. They passed the test for verification, despite being listed as misidentified cell lines by the International Cell Line Authentication Committee (ICLAC). Recombinant human TGF-α ~~and neuregulin 1β~~(100-16A) and neuregulinβ-1 (heregulinβ-1) (100-03) were purchased from PeproTech (Rocky Hill, NJ).

Real-time PCR (RT-PCR)

Total mRNA extraction was performed using the NucleoSpin®RNA II system~~.~~ (Macherey-Nagel AG, Oensingen SO, Switzerland). One 1 µg cDNA was prepared using the “High Capacity cDNA Reverse Transcription Kit” (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA). Gene expression was measured by the QuantStudio™ 6 real-time PCR system and QuantStudio software V1.2 (Thermo Fisher Scientific) using PowerUpTM SYBR Green Master Mix (A25741) (Applied Biosystems by Thermo Fisher Scientific). Relative gene expression was calculated using ~~a variation of~~ the 2^(-~~delta~~ delta CT) method (Livak & Schmittgen 2001). Hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*) expression was

selected for normalization. The following primers were used: HPRT1 (forward 5'-TGAGGATTTGGAAAGGGTGT-3', reverse 5'-GAGCACACAGAGGGCTAC AA-3'); *EGFR (*forward 5'-GAGTCGGGCTCTGGAGGAAA-3', reverse 5'-CAGTTATTGAACATCCTCTGG AC-3'~~);~~) (Felsberg et al. 2017, Weller et al. 2014);*ERBB2* (forward 5'-ATAGACACCAACCGCTCTCG-3', reverse 5'-ATCCTCAGAACTCTCTCCCCAG-3'); *ERBB3* (forward 5'-AACTCTCAGGCAGTGTGTCC-3', reverse 5'-AGCACAATCTCAAGGTTCCCC-3'); *ERBB4* (forward 5'-AGGATGTGGACGTTGCCATAAG-3', reverse 5'-ACCGTCCTTGTCAAAGTCTGG-3'); *EGF* (forward 5'-GTGTGCTGGACGCCTGTCT-3', reverse 5'-CTTACGGAATAGTGGTGGTCATCTT-3'): *HB-EGF* (forward 5'-TGGGCATGACTAATTCCCACTG-3', reverse 5'-AAGTCTTTCCCCTCTGCAGTC-3'); *TGF-α* (forward 5'-CCAGGTCCGAAAACACTGTGAG - 3', reverse 5'-AAACTCCTCCTCTGGGCTCTTC-3'); *EREG* (forward 5'-ATCACAGTCGTCGGTTCCAC-3', reverse 5'- CCATTCAGACTTGCGGCAAC-3'); *BTC* (forward 5'- GTGCAGCTACCACCACACAATC-3', reverse 5'-TTCATCACAGACACAGGAGGGC-3'); *NRG1* (forward 5'-TGTGTCTTCAGAGTCTCCCAT-3', reverse 5'- AAGCACTCCCCTCCATTCAC-3'); *NRG2* (forward 5'- GTGAGCACCACCCTGTCATC-3', reverse 5'-TGGACATTTGCAGGAGAGCTG-5'); *NRG3* (forward 5'-GCCCCAAATTTCATACGACGA-3', reverse 5'- TATGGGATCCGGTCAGGGTT-3'); *AREG* (5'- TTGATACTCGGCTCAGGCCA-3', reverse 5'-CCCCAGAAAATGGTTCACGC-3').

Immunoblot analysis

Total protein extracts were prepared using radio-immunoprecipitation assay (RIPA) lysis buffer (pH 7.8) containing 25 mM Tris-HCl, 120 mM NaCl, 5 mM EDTA and 0.5% NP-40 supplemented with 2 µg/mL aprotinin, 10 µg/mL leupeptin, 100 µg/mL phenylmethylsulfonyl fluoride, 200 mM sodium orthovanadate, ~~0.5 M NaF,~~ protease inhibitor cocktail ~~sets III and IV~~(P8340) and phosphatase inhibitor cocktails 2 (P5726)

and 3 (P0044) (Sigma Aldrich, St. Louis, MO). Nuclear and cytoplasmic sub-fractionation of cultured cells was performed by NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (78833, ThermoScientific, Waltham, MA).

Primary antibodies were as follows: rabbit anti-lamin B1 (1/2000, Ab 16048, RRID:AB_443298, Abcam, Cambridge, UK), rabbit anti-phospho-EGFR (Tyr 1068~~) (Cell Signaling Technology, CST, Danvers, Massachusetts~~), rabbit anti-EGFR ~~(Sc-03, Santa Cruz Biotechnology, Santa Cruz, CA),~~ rabbit anti-phospho-HER2/ERBB2 (Tyr1221/1222), rabbit anti- HER2/ERBB2, rabbit anti-phospho-HER3/ERBB3 (Tyr1289), rabbit anti-HER3/ERBB3 (all 1/1000, #8339, RRID:AB_10860426, Cell Signaling Technology, CST~~), and rabbit anti-LaminB1 (Ab 16048, 1/2000) (Abcam, Cambridge, UK).~~, Danvers, MA). The membranes were exposed to rabbit-anti goat IgG HRP antibody (1/5000, sc-2768, RRID:AB_656964, Santa Cruz Biotechnology) or to HRP-conjugated secondary rabbit antibody (~~Santa Cruz Biotechnology~~1/5000, #7074, RRID:AB_2099233, CST). Protein levels were quantified by NIH ImageJ densitometric software (http://rsb.info.nih.gov/ij/). Band intensity was normalized to actin ~~or GAPDH loading control.~~(1/1000, sc-1616, RRID:AB_630836, Santa Cruz Biotechnology) or GAPDH (1/1000, EB07069, RRID:AB_2247304, Everest Biotech Ltd, Bicester, UK) loading controls.

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Proliferation, clonogenicity and spherogenicity assays

Proliferation assays were performed in a 96-well format, seeding 5'000~~ 30'000 cells / well.~~ cells / well for LN-18, LN-428, LN-319, A172, U87MG, T98G and LN-229, 10'000-15'000 cells / well for D247MG, LN-308, T-325 and ZH-161, and 30'000 cells / well for T-269, S-24 and ZH-305. To assess colony formation of LTC or spherogenicity of GIC, cells were seeded at different densities (50-100 cells for LTC and 300 cells for GIC) in 96-well plates. LTC were allowed to attach in complete DMEM for 24 h. Then the medium was replaced by DMEM in the absence or presence of the corresponding agent. GIC were allowed to form spheres for 24 h in complete NB medium and then supplemented with drugs at the final concentrations, as indicated. After 72 h (proliferation assay), or 8-16 days (clonogenicity), or 20 days (spherogenicity), metabolic activity was assessed by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reduction.

Statistical analysis

Data reported here were derived from biological and technical replicates. Representative data of experiments, performed two to three times using triplicate wells for viability and proliferation assays and duplicate wells for RT-PCR, are shown. Quantitative data were expressed as the mean ± standard error of mean (SEM). The statistical analyses were performed by the one-way ANOVA or two-way ANOVA with Bonferroni's multiple comparison tests (GraphPad Software, La Jolla, CA). Pearson's correlation coefficient was used in order to calculate correlations between mRNA and protein data. A p-value of $p<0.05$ was considered statistically significant. This study was not pre-registered, does not need institutional approval, and no randomization or blinding was used.

Results

Expression and activity of EGFR ~~family members~~ in human glioma cells

We first determined *EGFR* mRNA expression in the cell line panel. It varied strongly among cell lines with a fold difference of 44.6 between D247MG and LN-319 among the LTC ~~or~~, and of 11.5 between T-269 and T-325 among the GIC~~. Expression models. There was no difference in expression~~ levels ~~did not generally differ~~ between pooled LTC and pooled GIC (Fig. 1A). Expression of the deletion variant, *EGFRvIII* ~~mRNA~~, was not ~~expressed~~detected in any cell line, ~~using~~but *EGFRvIII* mRNA was readily detected in EGFRvIII-transfected LN-229 cells (Fan *et al.* 2013) which were used as a positive control (data not shown). EGFR protein was detected in all cell lines~~. pEGFR^{Tyr1068}~~, phosphorylated EGFR (pEGFR) was also detected ~~as 3 immunoreactive bands between 100 and 260 kDa~~ in ~~many, but not~~ all cell lines ~~(Fig. 1B). The signal specificity was confirmed by demonstrating that the immunoreactive bands decreased in response to increasing concentrations of gefitinib (Fig. 1D). The majority of pEGFR was localized in the nuclear compartment (Fig.~~, albeit at very low levels in LN-319.~~1C).~~ Quantification of protein bands using densitometry indicated that relative to total EGFR protein levels, pEGFR levels ~~in GIC~~ were higher in GIC than in LTC, with a pEGFR/EGFR ratio of 1~~.62~~ in GIC versus 0.44~~8~~ in LTC (Fig. 1B~~) which may be~~). This is likely due to EGF supplementation in the GIC culture medium. The signal specificity for EGFR was confirmed by a decrease in response to the EGFR TKI gefitinib (Fig. 1C). ~~Overall, there was no~~ There was overall no correlation between *EGFR* mRNA ~~and EGFR protein and only a trend for negative correlation between~~, total EGFR protein and pEGFR (Table ~~S1).~~S2), suggesting the existence of regulatory pathways of EGFR phosphorylation beyond *EGFR* gene expression. ~~To better understand the differences among pEGFR levels in the cell line panel,~~

Expression of ERBB receptor family members in human glioma cells

Next we determined the expression of ERBB family members ~~(Fig. 2A-E) and their ligands (Fig. 3).~~in the glioma cell line panel. *ERBB2* mRNA was expressed in all cell lines except ZH-161 ~~(Fig. 2A). The~~. By far the highest *ERBB3* mRNA level was found in LN-229 whereas LN-428, D247MG, LN-308, S-24 and ZH-305 had low levels, and T98G was negative ~~(Fig. 2B).~~. *ERBB4* mRNA was not detectable in the half of the cell line panel and ~~varied considerably between very~~detected at low ~~and~~to moderate expression levels among the other cell lines ~~(Fig. 2C). mRNA and protein levels~~

~~correlated for ERBB2 and ERBB3 (Fig. 2A,B,D,E, Table S1).~~. ERBB2 protein varied considerably among cell lines whereas ERBB3 protein was detected at high levels in LN-229 cells only (Fig. 2). ERBB4 protein was not detected in any of glioma cell lines (data not shown). mRNA and protein levels correlated for ERBB2 and ERBB3 (Table S2). Compared with the cell line panel, TCGA analysis disclosed a similar relative abundance of *ERBB* mRNA species in human glioblastomas *in vivo*~~. It indicated~~ and revealed trend for correlation between high expression levels and inferior survival for *EGFR* and *ERBB2*, but not *ERBB3* or *ERBB4* (Fig. S1).

Expression of ERBB ligands in human glioma cells

Next we determined the expression of ERBB family ligands in the glioma cell line panel. S-24 was the only *EGF* mRNA-negative cell line. At mRNA levels, *EREG* (in some cell lines), *TGF-α* and *NRG1* were the dominant ligands. T98G was the only *TGF-α*-negative cell line. High *EREG* mRNA expression was detected in LN-18, U87MG and T-325 cells. *BTC* showed low or no expression in most of the cell lines. GIC had lower *NRG1* mRNA expression than LTC, LN-308 was the only one LTC with low *NRG1* mRNA. Low *NRG2* mRNA expression was detected only in LN-319 and LN-308, the other cell lines showed higher mRNA levels. ZH-305 was the only *NRG3* mRNA~~-~~-positive cell line. ZH-161 and ZH-305 were the only *AREG*~~-~~-positive cell lines, yet with very low expression (Fig. 3). ~~EGFR ligand~~There were correlations between *EREG* and *BTC* as well as between *EGF* and *NRG1* gene expression ~~appeared to be co-regulated within the GIC panel, but not~~(Table S3). Expression of *NRG1*, an exclusive ERBB3 and ERBB4 ligand, correlated with *ERBB1* mRNA, pEGFR in LTC ~~(Table S2). Notably~~and with *ERBB2* mRNA. Similarly, *TGF-α*, an EGFR specific ligand, correlated strongly with ERBB2 protein level as well as with ERBB3 mRNA expression ~~correlated with the levels of EGFR~~ and ~~ERBB2~~ protein (Table ~~S3~~S4). TCGA analysis showed similar relative expression levels of ERBB ligands, e.g., low level expression of *AREG* and *NRG2-4*, and revealed no major correlation between expression levels and survival (Fig. S2).

Biological activity of EGFR signaling in maintaining the GIC phenotype

Next~~,~~ we analyzed glioma cell sensitivity to different anti-EGFR agents, gefitinib, a TKI of EGFR, and erbitux, ~~an~~a neutralizing antibody to EGFR. Neither LTC nor GIC were particularly sensitive to these agents in 72 h continuous exposure assays (Table 1, Fig. S3). In order to investigate the role of ERBB ligand family members for

sphere formation, limiting dilution assays were performed in four GIC lines. Omission of EGF from the medium expectedly abrogated sphere formation in all GIC. Accordingly, gefitinib decreased spherogenicity ~~(Fig. 4).~~, too. Replacement of EGF by TGF-α maintained sphere formation (Fig. 4). In contrast, neuregulin-~~-~~1 had no such effect (data not shown). Accordingly, exposure to exogenous EGF or TGF-α induced pEGFR levels strongly, whereas withdrawal of EGF or exposure to gefitinib abrogated EGFR phosphorylation (Fig. 4E), corroborating the spherogenicity data.

Nuclear localization and subcellular regulation of ERBB receptors

It has been previously shown that EGFR can translocate from the membrane to the nucleus and may contribute to drug resistance (Brand *et al.* 2011, Li *et al.* 2009, Fan et al. 2013). Therefore, we performed subcellular fractionation for all LTC and GIC in order to assess nuclear versus cytoplasmic fractions of phosphorylated and total EGFR. Unexpectedly, the majority of pEGFR was localized in the nuclear compartment (Fig. 5A). Both nuclear and cytoplasmic EGFR were simultaneously decreased when ZH-161 and S-24 cells were exposed to gefitinib. Moreover, gefitinib treatment prior to EGF stimulation prevented nuclear and cytoplasmic pEGFR induction by subsequent stimulation (Fig. 5B). Interestingly, nuclear localization was also observed for ERBB2 and ERBB3 in LN-229 cells. pERBB2, and less so pERBB3, increased in response to EGF, but only in the cytoplasmic compartment. pERBB2 was decreased by gefitinib in both fractions, although less so in the nuclear compartment, whereas cytoplasmic pERBB3 strongly increased in response to gefitinib (Fig. 5C).

Discussion

~~The *EGFR* gene is the most commonly amplified and overexpressed proto-oncogene and a frequent mutational target in glioblastoma. *EGFR* gene amplification is detectable in approximately 40% of all glioblastomas and common in the classic or receptor tyrosine kinase (RTK) type 2 molecular subtype of isocitrate dehydrogenase-wildtype glioblastoma.~~ The present comprehensive characterization of expression and biological activity of ERBB family receptors and their ligands in a large panel of glioma models was conducted because of the increasing interest in targeting these molecules for the treatment of glioblastoma. Such treatment approaches directed mainly to EGFR (ERBB1), but also to HER2 (ERBB2) include tyrosine kinase inhibitors, unarmed antibodies, antibody drug conjugates, vaccines and even cellular immunotherapies (~~Sturm~~Weller et al. ~~2012, Brennan~~2017b, Sampson et al. ~~2013~~2014)~~. Half of *EGFR* amplified glioblastomas carry a tumor-specific deletion variant (EGFRvIII) that is~~ , highlighting the urgent need to better understand this receptor ligand system in glioblastoma.

~~characterized by an in-frame deletion of exons 2-7, resulting in constitutive EGFR activation (Felsberg *et al.* 2017). The failure of EGFR TKI to improve outcome has been attributed to insufficient target coverage, e.g., because of limited blood brain barrier penetration or the redundance of TK-dependent signalling pathways in glioblastoma or both (Furnari *et al.* 2015).~~

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~~Here, we explored expression and biological activity of EGF/EGFR-related ligands and receptors which are known to exhibit potential for cross-talk with the EGFR sigaling pathway in a panel of human glioma cell lines, including GIC models.~~ We find that the EGFR pathway ~~shows heterogeneous patterns~~is activated as confirmed by the detection of ~~activation~~pEGFR in almost all glioma ~~cell lines~~models *in vitro* (Fig. 1). ~~The interpretation of cell culture data on the biological role of the EGFR pathway is limited by the fact~~This was surprising since it has previously been observed that ~~EFGR~~*EGFR* amplification ~~including~~ and in particular expression of the *EGFRvIII* ~~expression is~~deletion mutation are commonly lost upon prolonged glioma cell maintenance in culture (Pandita *et al.* 2004, Bigner *et al.* 1990).

EGF and related ligands are present in the FCS which is used to maintain the LTC in culture ~~(Pandita *et al.* 2004, Bigner *et al.* 1990), and that~~over years, but pEGFR was maintained in most models even under the experimental conditions of serum-free culture used in Fig. 1. In contrast, supplementing EGF is essential in most contemporary paradigms of maintaining GIC cultures~~, as confirmed here in Fig. 4.~~ (Lathia *et al.* 2015) and likely accounts for the relatively higher pEGFR levels in GIC models than in LTC models (Fig. 1).

Beyond EGFR, cultured glioma cells also express other ERBB family members, notably ERBB2 and ERBB3, and exhibit ERBB phosphorylation, suggesting pathway activity, but in a highly cell line-specific manner (Fig. ~~2).~~2). The relative expression levels allow to speculate that GIC preferentially signal via EGFR dimers or EGFR/ERBB2 heterodimers whereas the widely used LN-229 model may also signal via ERBB2/ERBB3 heterodimers since both were expressed relatively high relative to EGFR. Importantly, expression patterns in the cell line panel resembled expression data deposited in the TCGA database (Fig. S1~~). ~~), suggesting that such cell culture models may be appropriate tools to explore ERBB family molecule-directed therapies.

There was also widespread expression of *EGF* family ligands in the glioma cell line panel (Fig. 3~~), and some~~) that resembled patterns of expression in the TCGA database (Fig. S2). Some of these genes appeared to be transcriptionally co-regulated (Tables ~~S2, S3). Finally, there~~S3, S4), indicating up-stream common regulatory pathways that might be better amenable to therapeutic intervention. There is to date only limited data on the biological role of EGF-related growth factors in maintaining the malignant phenotype of gliomas. EGF and FGF are typically included in the cell culture medium used to generate patient-derived glioma cell lines directly *ex vivo*. In that regard, we observed that ~~at least~~ TGF-α, which acts on EGFR, but not NRG1, which only acts on ERBB3 and ERBB4, may well compensate for EGF in ~~situations at the level~~terms of EGFR phosphorylation and sphere formation where EGF supply is suppressed (Fig. ~~4).~~4). The almost universal resistance of glioma models *in vitro* to clinically relevant concentrations of gefitinib or erbitux (Table 1) precluded an analysis of patterns of receptor and ligand expression associated with sensitivity or resistance to EGFR targeting.

Once activated by phosphorylation, EGFR not only induces down-stream signaling, but also enters the cytosol. Several lines of evidence indicates that a significant

amount of EGFR enters the nucleus of glioma cells to exert specific functions (Burel-Vandenbos *et al.* 2013, Fan et al. 2013), e.g., promoting transcription of the antiapoptotic BCL-XL gene (Latha *et al.* 2013) or modulating DNA repair (Dittmann *et al.* 2005). It is also believed that nuclear EGFR can retain its tyrosine kinase activity and phosphorylate further substrates (Wang *et al.* 2006). Our analyses confirm the accumulation of pEGFR in the nucleus (Fig. 5). We observed that either EGF stimulation or gefitinib treatment increased or decreased cytoplasmic as well as nuclear pEGFR simultaneously (Fig. 5B, C), suggested activity at both sites or rapid cycling. Importantly, besides EGFR, ERBB2 and ERBB3 may also exhibit nuclear localization. Both may contribute to evasive resistance to EGFR targeting by TKI since pERBB2 in the nucleus was poorly responsive to gefitinib and since pERBB3 even paradoxically increased in both compartments (Fig. 5C). Nuclear localization of ERBB3 was previously reported in breast cancer cells (Offterdinger *et al.* 2002), but these authors observed a lack of nuclear ERBB2 and absent nuclear phosphorylation of ERBB3. Sustained phosphorylation of ERBB3 can be explained by the presence of nuclear ERBB2 once pEGFR is decreased upon gefitinib treatment. Overexpression of ERBB ligands has been linked to nuclear translocation of EGFR in non-small cell lung cancer cells (Li et al. 2009) and its

nuclear accumulation has been suggested to be in part responsible for intrinsic resistance to inhibitors targeting EGFR at the cell surface. Sustained nuclear pERBB2 and increased cytoplasmic phosphorylation levels of ERBB3 as observed in LN-229 cells (Fig. 5) warrants efforts to develop therapies targeting multiple ERBB molecules.

Despite disappointing results with EGFR TKI or EGFRvIII-directed vaccines (Weller *et al.* ~~2017~~2017a, Furnari et al. 2015), targeting EGFR as one of the dominant molecular lesions in glioblastoma will continue to be studied, with antibody drug conjugates such as ABT-414 being the most promising approach at present ~~(van den Bent *et al.* 2017)~~(van den Bent *et al.* 2017, Gan et al. 2017). A better understanding of the role of other ERBB family ligands and receptors in modulating constitutive or acquired resistance to EGFR targeting in glioblastoma should help to guide therapeutic strategies and to potentially enrich for patient populations most likely to derive benefit. The present dataset will facilitate the choice of appropriate cell culture models for future studies on ERBB family protein-directed therapies in glioblastomas.

Competing interest

This work was supported by an unrestricted research grant from Abbvie to M.W..
M.W. has received further research grants from Acceleron, Actelion, Bayer, Merck, Sharp & Dohme (MSD), Merck (EMD), Novocure, OGD2, Piqur, Roche and Tragara, and honoraria for lectures or advisory board participation or consulting from Abbvie, BMS, Celgene, Celldex, Merck, Sharp & Dohme (MSD), Merck (EMD), Novocure, Orbus, Pfizer, Progenics, Roche, Teva and Tocagen, all unrelated to this work. C.v.A. and E.S. declare that they have no conflict of interests.

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Table 1. Growth inhibitory effects of gefitinib and erbitux in glioma cells *in vitro*.*

EC50 (µM) Metabolic activity (72 h)		
Cell	**Gefitinib**	**Erbitux**
LN-18	**> 10 µM**	**> 100 µg/ml**
LN-428	**> 10 µM**	**> 100 µg/ml**
D247MG	**> 10 µM**	**Nd**
LN-319	**> 10 µM**	**> 100 µg/ml**
A172	**> 10 µM**	**> 100 µg/ml**
U87MG	**> 10 µM**	**> 100 µg/ml**
T98G	**> 10 µM**	**> 100 µg/ml**
LN-308	**> 10 µM**	**> 100 µg/ml**
LN-229	**6 µM**	**> 100 µg/ml**
T-325	**> 10 µM**	**> 100 µg/ml**
T-269	**10 µM**	**> 100 µg/ml**
ZH-161	**> 10 µM**	**> 100 µg/ml**
S-24	**0.3 µM**	**> 100 µg/ml**
ZH-305	**> 10 µM**	**Nd**

*EC50 values for growth inhibition were determined by MTT assay at 72 h exposure (n=4 technical replicates).

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Figure legends

Figure 1. **Characterization of ~~LTC and GIC~~the long-term glioblastoma cell line ~~panel~~(LTC) and glioma-initiating cell line (GIC) panels for EGFR expression and ~~activity.~~phosphorylation.** A,B. The cells were assessed for mRNA expression of epidermal growth factor receptor (*EGFR ~~(~~/ERBB1)* (~~A~~representative results of at least 2 biological replicates are depicted with n=2 technical replicates) by RT-PCR~~,~~ (A), as well as for ~~total and~~ phosphorylated and total EGFR protein ~~levels~~ in total cell lysates by immunoblot (representative results of 2 biological replicates) (B). C. ~~Cells were also evaluated for the subcellular localization of phosphorylated and total EGFR protein levels in different cellular compartments by using nuclear (N) and cytoplasmic/membrane (C) fractionation. D.~~ LN-229, T-269 or S-24 cells were exposed to 0.1, 1 or 10 µM gefitinib for 24 h and pEGFR levels were assessed by immunoblot~~.~~ (representative results of n=2 biological replicates). Actin (B~~,C~~) or GAPDH (~~D) served~~C) were used as loading controls~~, lamin B1 was used as membrane fraction loading control (C).~~.

Figure 2. **ERBB receptor family expression and activity.** A-E. The cells were assessed for *ERBB2*, *ERBB3* and *ERBB4* gene expression (~~A-C~~representative results of at least 2 biological replicates are depicted with n=2 technical replicates) by RT-PCR~~,~~ (A-C), as well as for ~~total and~~ phosphorylated and total protein levels by immunoblot (n=1) (D,E). Actin served as a loading control~~.~~ (D,E).

Figure 3. **ERBB ligand expression**. Quantification of the ERBB ligand mRNA expression by RT-PCR: epidermal growth factor (*EGF~~,~~)*, heparin-binding epidermal growth factor (*HB-EGF~~,~~)*, transforming growth factor alpha (*TGF-α~~,~~)*, epiregulin (*EREG~~,~~)*, betacellulin (*BTC~~,~~)*, neuregulin (*NRG1-3)* and amphiregulin (*AREG~~.~~)* (representative results of at least 2 biological replicates are depicted with n=2 technical replicates).

Figure 4. **ERBB ligand rescue upon epidermal growth factor (EGF) withdrawal-mediated loss of glioma-initiating cell lines (GIC) sphere formation.** T-325 (A), ZH-161 (B), S-24 (C) or ZH-305 (D) cells were seeded at densities of 30, 100 or 300 cells per well in 96 well plates in medium containing EGF (20 ng/ml) plus fibroblast

growth factor (FGF) (20 ng/ml), FGF (20 ng/ml) only, transforming growth factor alpha (TGF-α) (60 ng/ml) plus FGF (20 ng/ml), or EGF (20 ng/ml) plus FGF (20 ng/ml) plus gefitinib (10 μM). Metabolic activity was assessed by MTT assay after 20 days. Data are expressed as mean ± SEM (**p<0.01, ***p<0.001: + EGF/+FGF vs - EGF/+FGF, $^{\#}$p<0.05, $^{\#\#\#}$p<0.001: - EGF/+FGF vs -EGF/+FGF/+TGF-α, $^{\phi\phi}$p<0.01, $^{\phi\phi\phi}$p<0.001: +EGF/+FGF vs +EGF/+FGF/+gefitinib~~). E. pEGFR~~, two-way ANOVA with Bonferroni correction) (representative results of 2 biological replicates are depicted with n=2 technical replicates). E. Phosphorylated epidermal growth factor receptor (pEGFR) levels were determined at 24 h without or with EGF (50 ng/ml, 15 min) or TGF-α (150 ng/ml, 15 min) exposure, as well as with gefitinib (10 μM) treatment, by immunoblot~~.~~ (n=1). GAPDH served as loading control.

Figure 5. **Cytoplasmic and nuclear localization of ERBB receptors.** Cells were also evaluated for the subcellular localization of phosphorylated and total EGFR protein levels by using nuclear (N) and cytoplasmic (C) fractionation (A). B. ZH-161 or S-24 were either deprived of EGF for 24 h, or pretreated with 10 μM gefitinib for 24 h, and were then stimulated with EGF (50 ng/ml) for 15 or 60 min. C. LN-229 cells were pretreated with gefitinib and stimulated with EGF as described in B and assessed for pERBB1/ERBB1, pERBB2/ERBB2 and pERBB3/ERBB3 (n=1). GAPDH served as loading control, lamin B1 was used as nuclear fraction loading control.

Figure 1

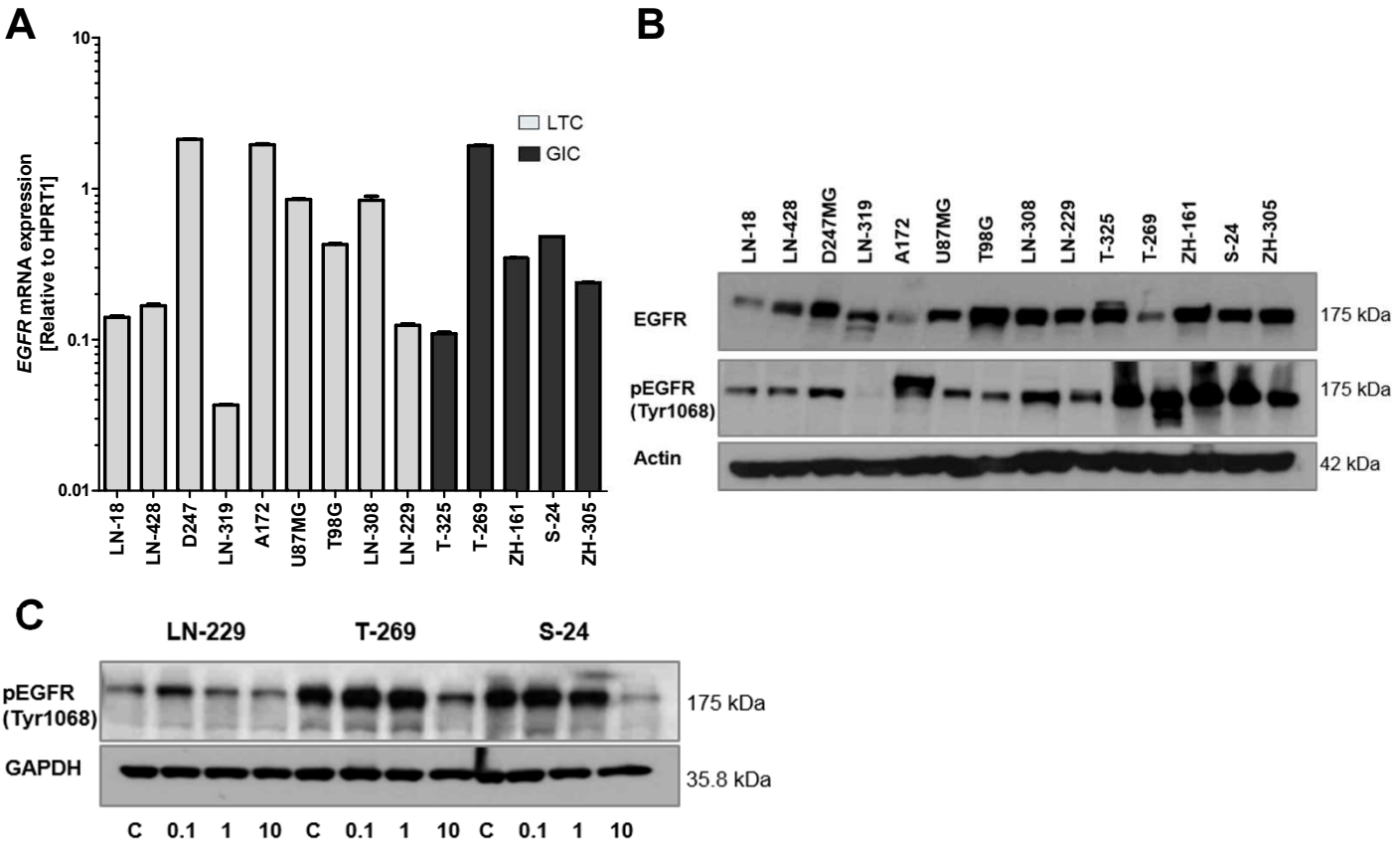

A
10
1
0.1
0.01
EGFR mRNA expression
[Relative to HPRT1]
LTC
GIC
LN-18
LN-428
D247
LN-319
A172
U87MG
T98G
LN-308
LN-229
T-325
T-269
ZH-161
S-24
ZH-305
B
LN-18
LN-428
D247MG
LN-319
A172
U87MG
T98G
LN-308
LN-229
T-325
T-269
ZH-161
S-24
ZH-305
EGFR
175 kDa
pEGFR
(Tyr1068)
175 kDa
Actin
42 kDa
C
LN-229
T-269
S-24
pEGFR
(Tyr1068)
175 kDa
GAPDH
35.8 kDa
C 0.1 1 10 C 0.1 1 10 C 0.1 1 10

Figure 2

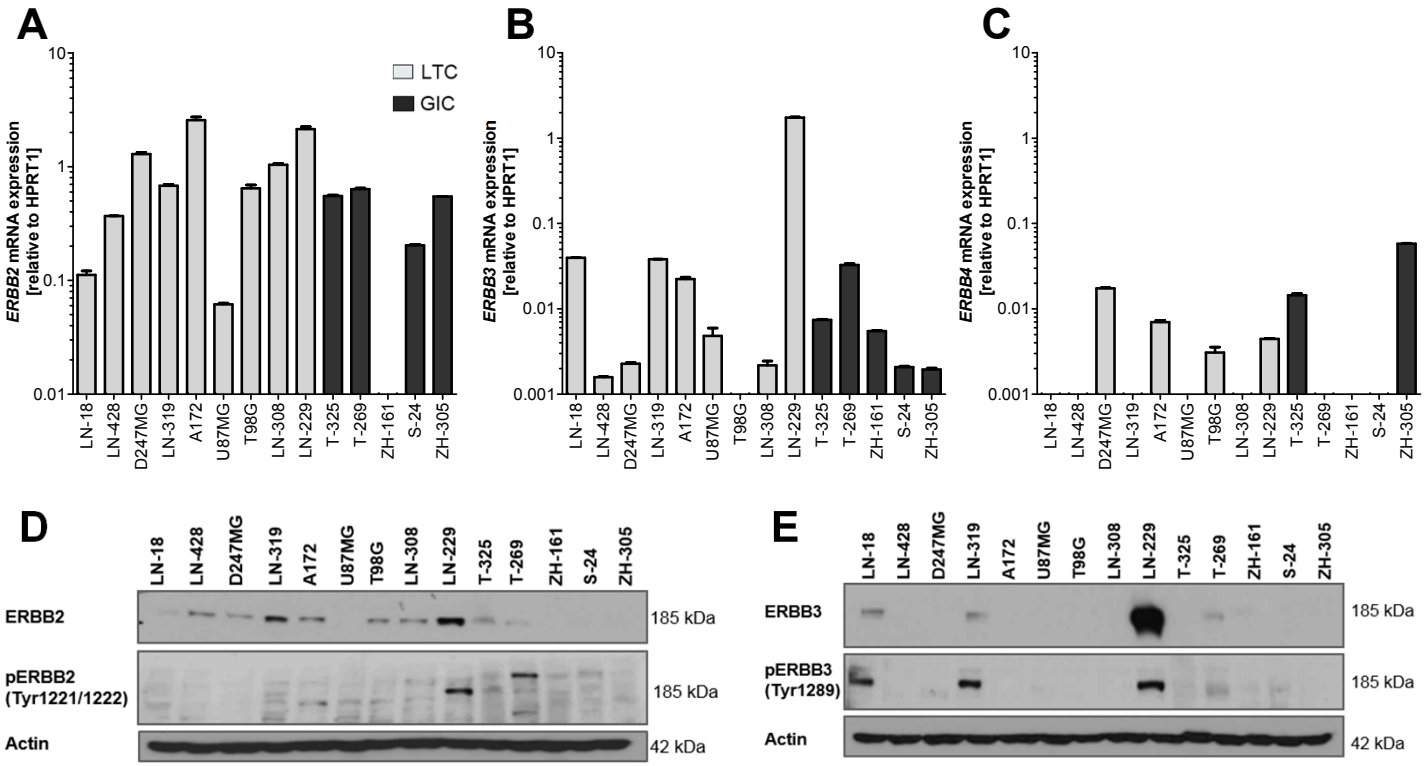

A
B
C
LTC
GIC
ERBB2 mRNA expression [relative to HPRT1]
ERBB3 mRNA expression [relative to HPRT1]
ERBB4 mRNA expression [relative to HPRT1]
10
1
0.1
0.01
0.001
LN-18
LN-428
D247MG
LN-319
A172
U87MG
T98G
LN-308
LN-229
T-325
T-269
ZH-161
S-24
ZH-305
D
E
ERBB2
pERBB2 (Tyr1221/1222)
Actin
ERBB3
pERBB3 (Tyr1289)
185 kDa
42 kDa

Figure 3

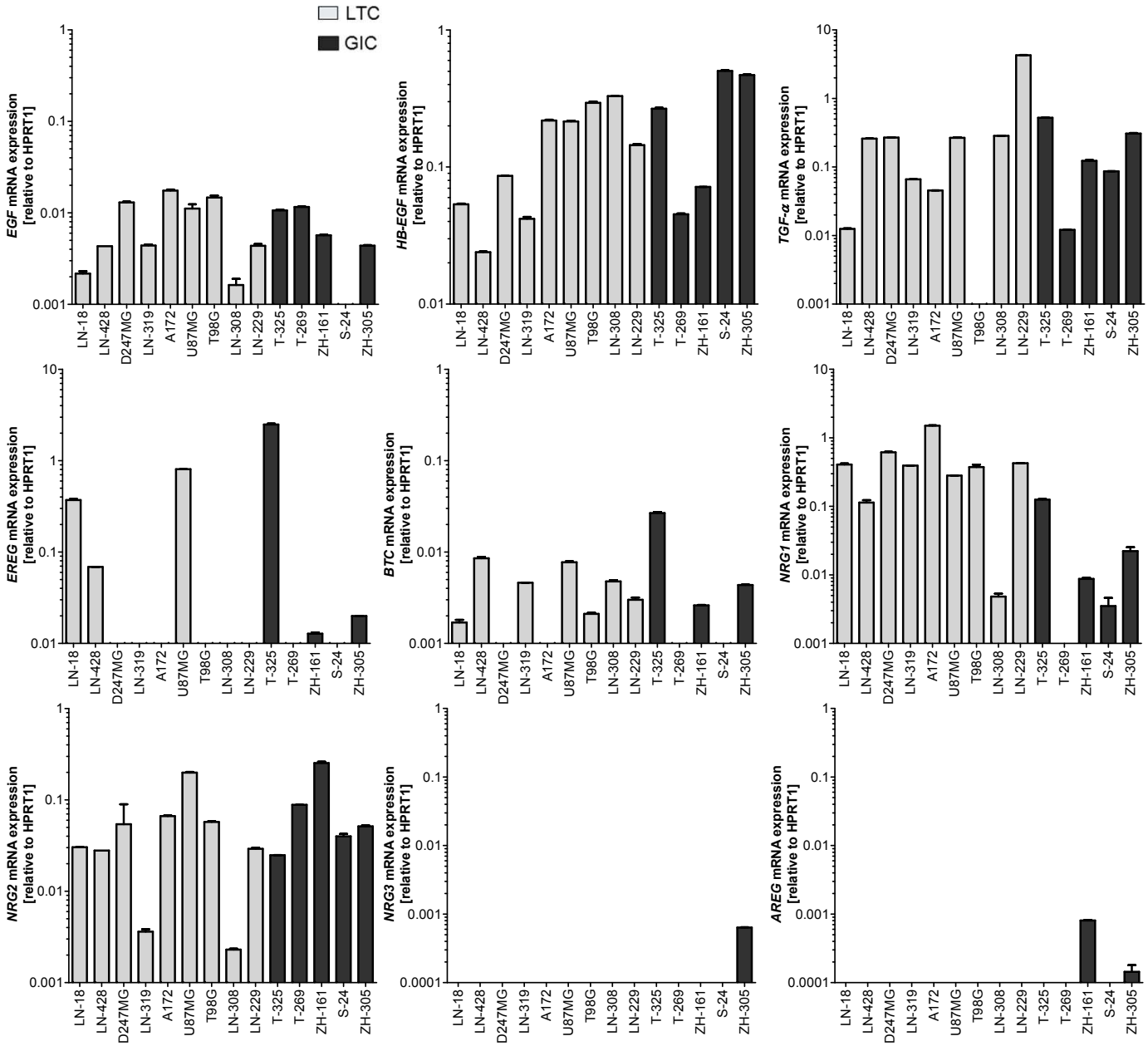

LTC
GIC
EGF mRNA expression [relative to HPRT1]
HB-EGF mRNA expression [relative to HPRT1]
TGF-α mRNA expression [relative to HPRT1]
EREG mRNA expression [relative to HPRT1]
BTC mRNA expression [relative to HPRT1]
NRG1 mRNA expression [relative to HPRT1]
NRG2 mRNA expression [relative to HPRT1]
NRG3 mRNA expression [relative to HPRT1]
AREG mRNA expression [relative to HPRT1]
LN-18
LN-428
D247MG
LN-319
A172
U87MG
T98G
LN-308
LN-229
T-325
T-269
ZH-161
S-24
ZH-305

Figure 4

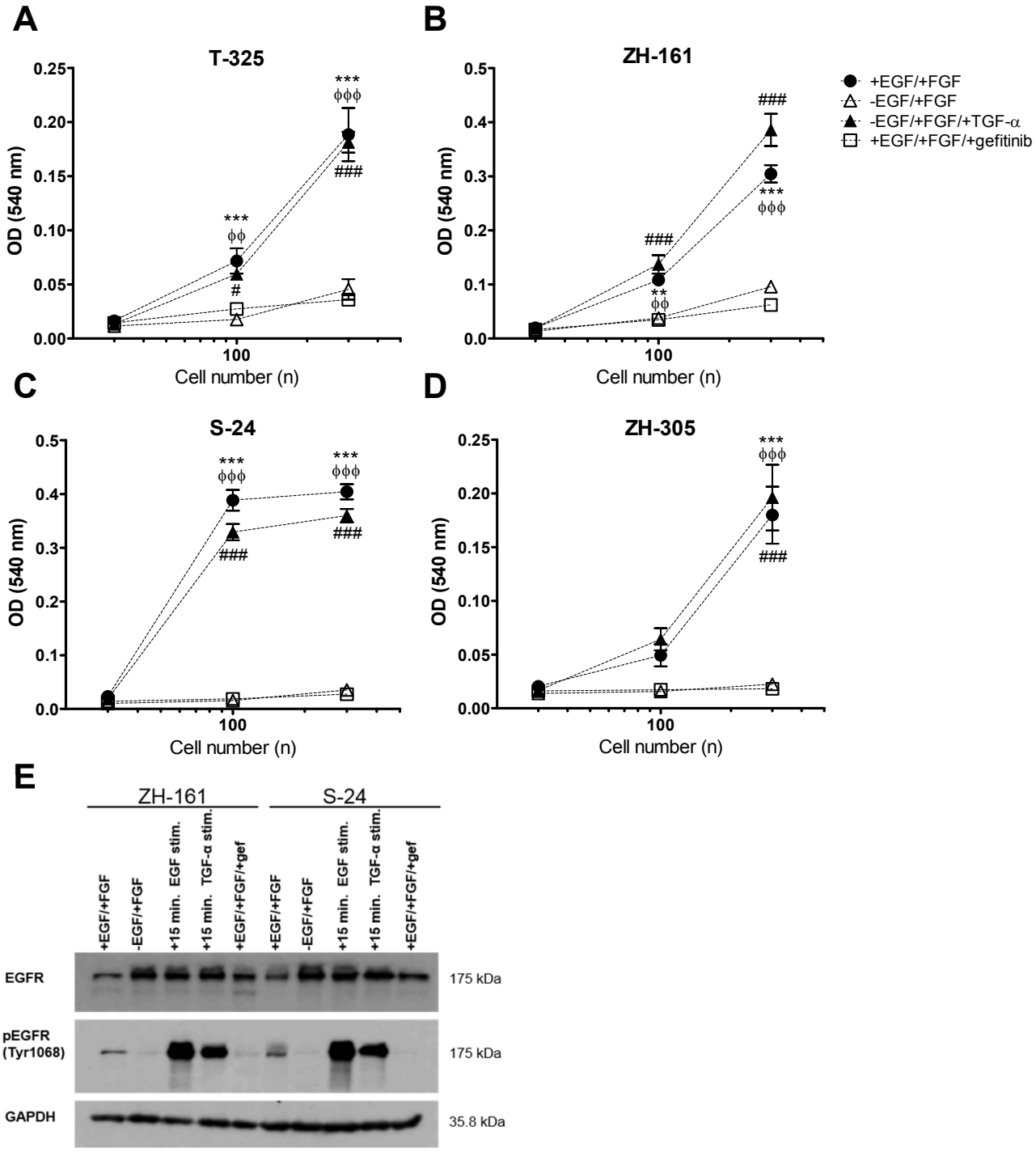
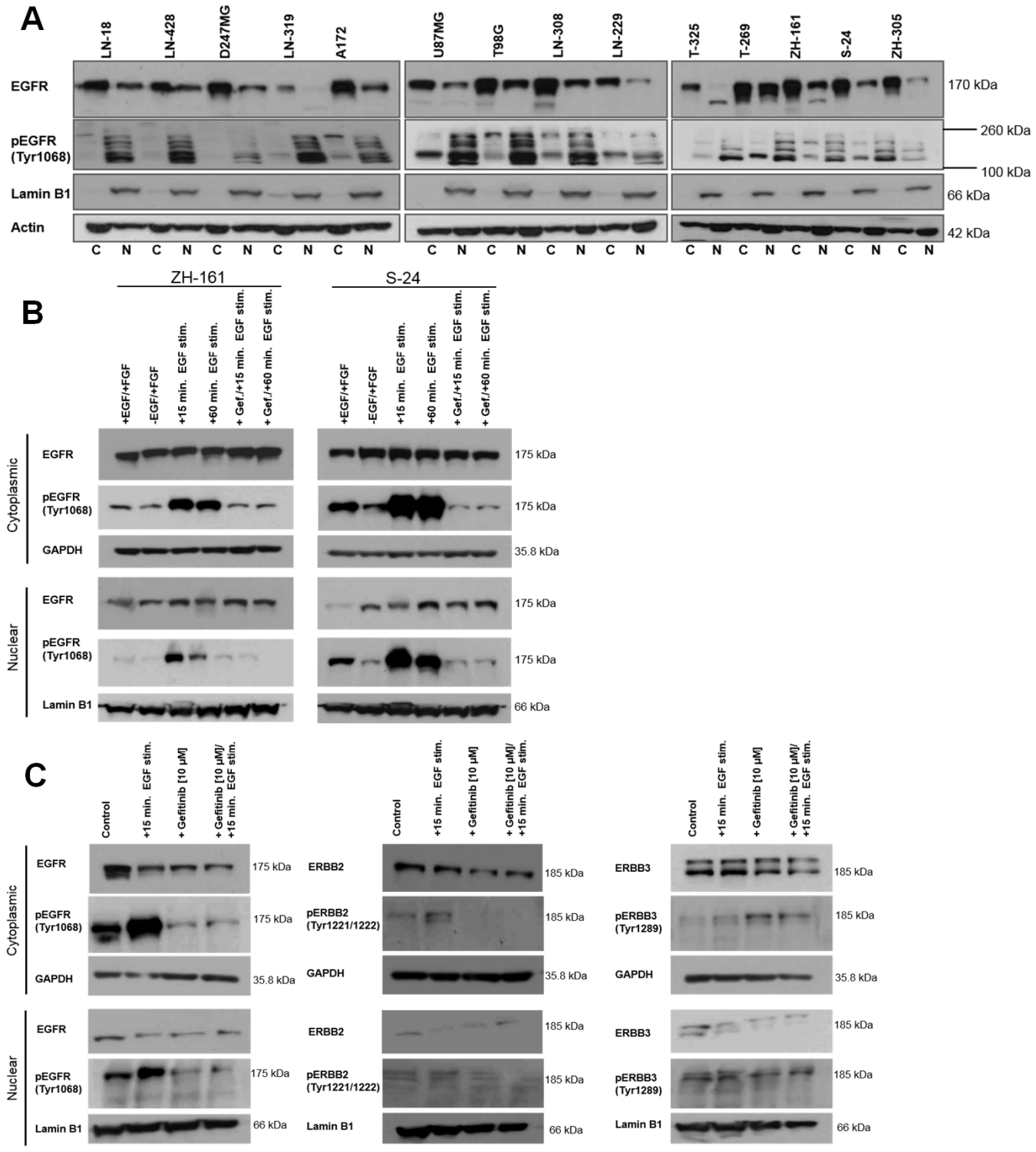
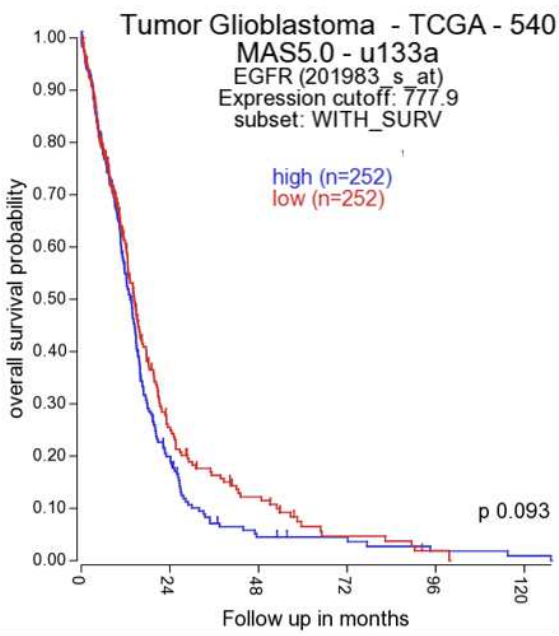

A
T-325
B
ZH-161
C
S-24
D
ZH-305
OD (540 nm)
Cell number (n)
100
+EGF/+FGF
-EGF/+FGF
-EGF/+FGF/+TGF-α
+EGF/+FGF/+gefitinib
E
ZH-161
S-24
+EGF/+FGF
-EGF/+FGF
+15 min. EGF stim.
+15 min. TGF-α stim.
+EGF/+FGF/+gef
EGFR
pEGFR (Tyr1068)
GAPDH
175 kDa
175 kDa
35.8 kDa

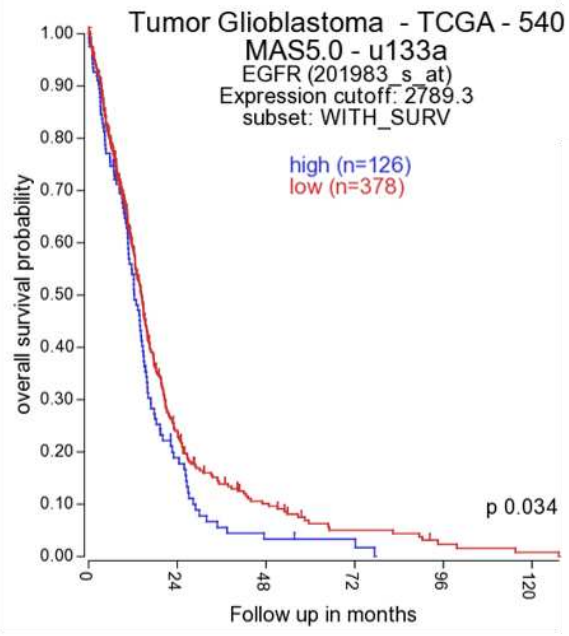
Figure 5

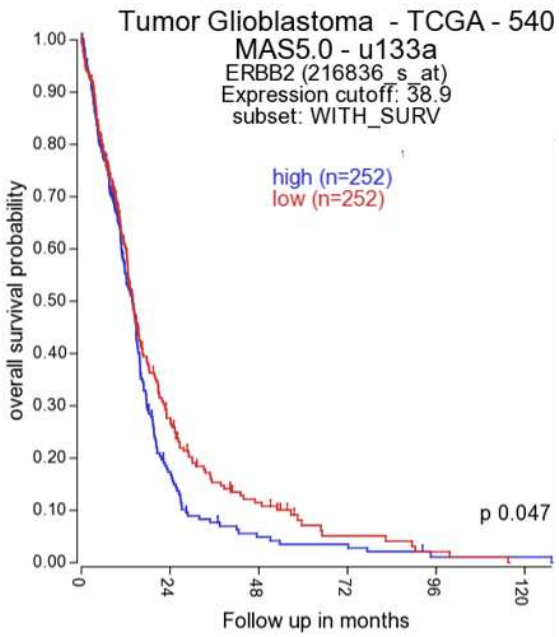

A
LN-18
LN-428
D247MG
LN-319
A172
U87MG
T98G
LN-308
LN-229
T-325
T-269
ZH-161
S-24
ZH-305
EGFR
pEGFR (Tyr1068)
Lamin B1
Actin
170 kDa
260 kDa
100 kDa
66 kDa
42 kDa
C N C N C N C N C N C N C N C N C N C N C N C N C N C N C N
B
ZH-161
S-24
+EGF/+FGF
-EGF/+FGF
+15 min. EGF stim.
+60 min. EGF stim.
+ Gef./+15 min. EGF stim.
+ Gef./+60 min. EGF stim.
Cytoplasmic
Nuclear
EGFR
pEGFR (Tyr1068)
GAPDH
Lamin B1
175 kDa
35.8 kDa
66 kDa
C
Control
+15 min. EGF stim.
+ Gefitinib [10 µM]
+ Gefitinib [10 µM]/ +15 min. EGF stim.
ERBB2
pERBB2 (Tyr1221/1222)
ERBB3
pERBB3 (Tyr1289)
185 kDa

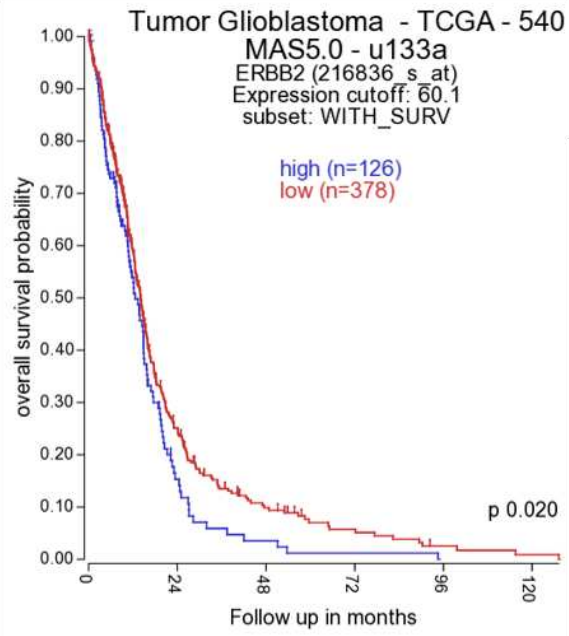
Epidermal growth factor receptor and ligand family expression and activity in glioblastoma

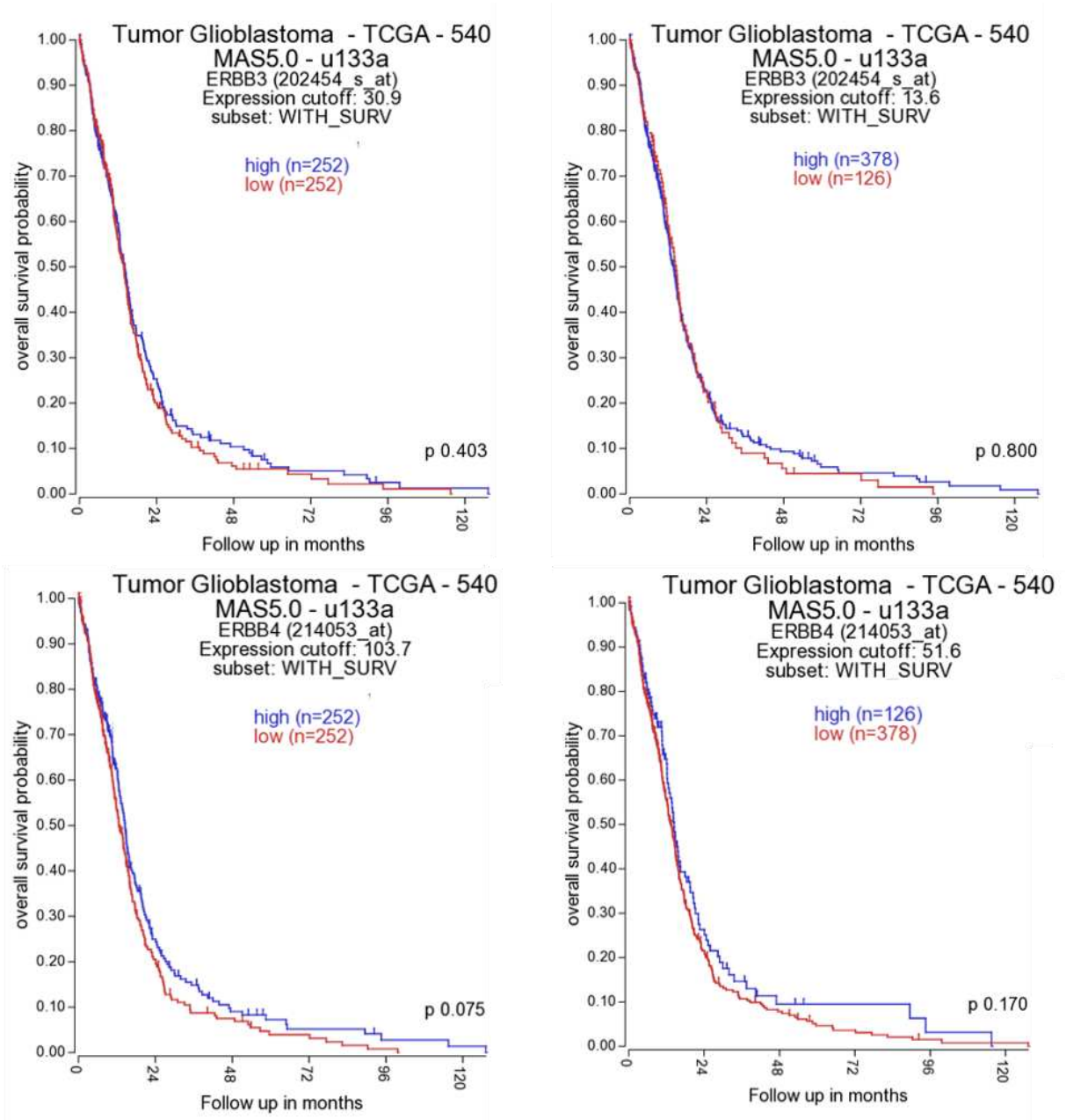
Caroline von Achenbach, Michael Weller, Emese Szabo



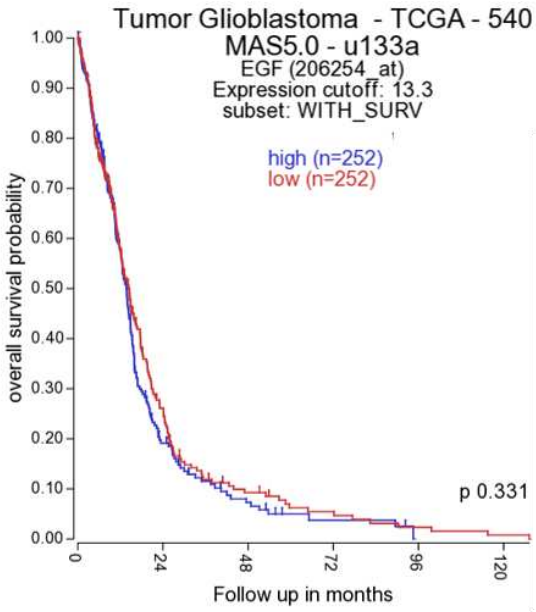


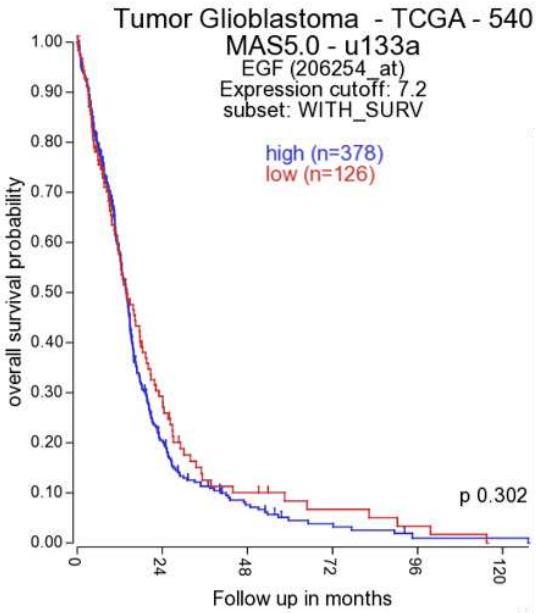


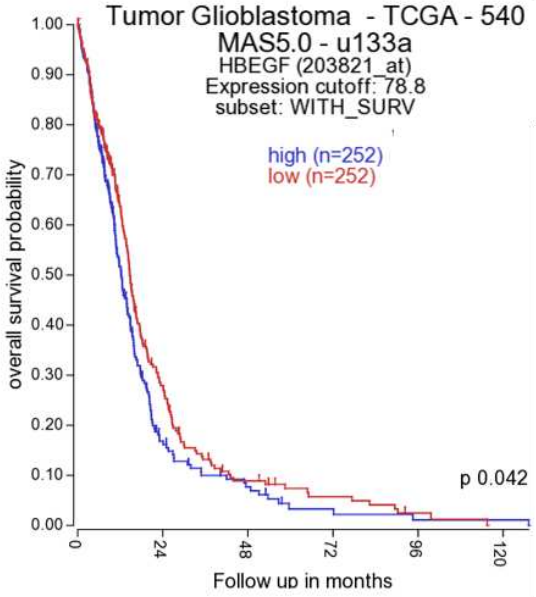


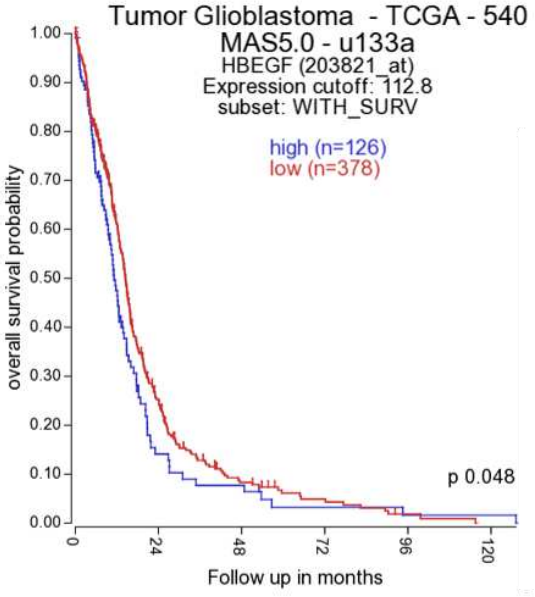


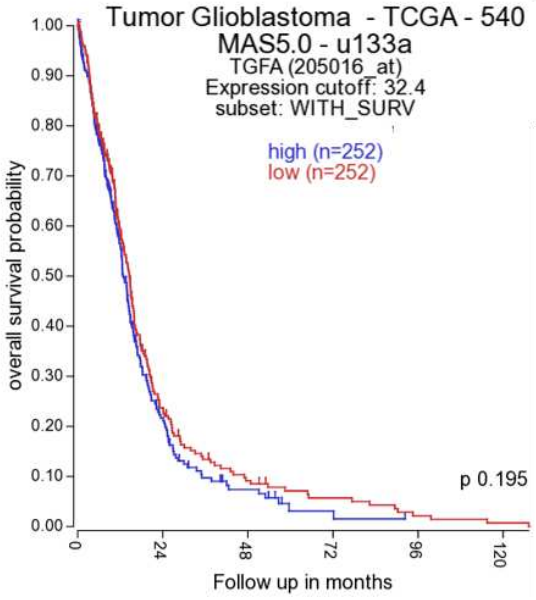
Supplementary Figure 1. **Association of gene expression levels of *ERBB* family receptors with survival in glioblastoma patients.** Kaplan Meier survival curves comparing gene expression status (high or low) are shown within the glioblastoma data set of the Cancer Genome Atlas network (TCGA) database using 'R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)' based on median (left panels) or optimum with limitations of quartile normalization (right panels) cut-off modus. Statistical significances (log-rank test) are indicated.

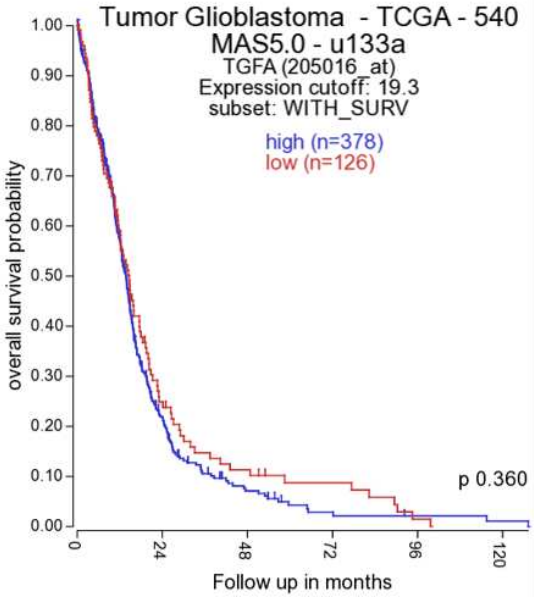

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
EGF (206254_at)
Expression cutoff: 13.3
subset: WITH_SURV
high (n=252)
low (n=252)
p 0.331
overall survival probability
Follow up in months

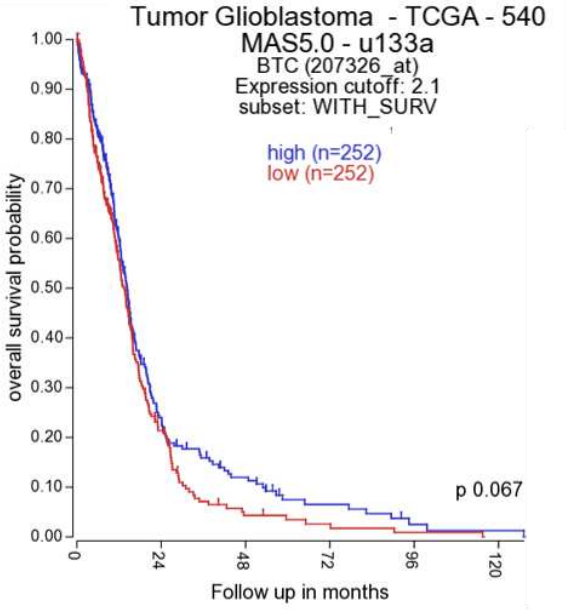

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
EGF (206254_at)
Expression cutoff: 7.2
subset: WITH_SURV
high (n=378)
low (n=126)
p 0.302
overall survival probability
Follow up in months

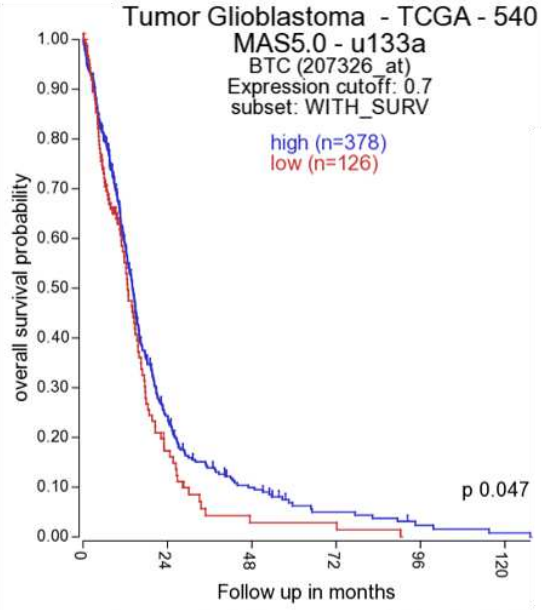

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
HBEGF (203821_at)
Expression cutoff: 78.8
subset: WITH_SURV
high (n=252)
low (n=252)
p 0.042
overall survival probability
Follow up in months

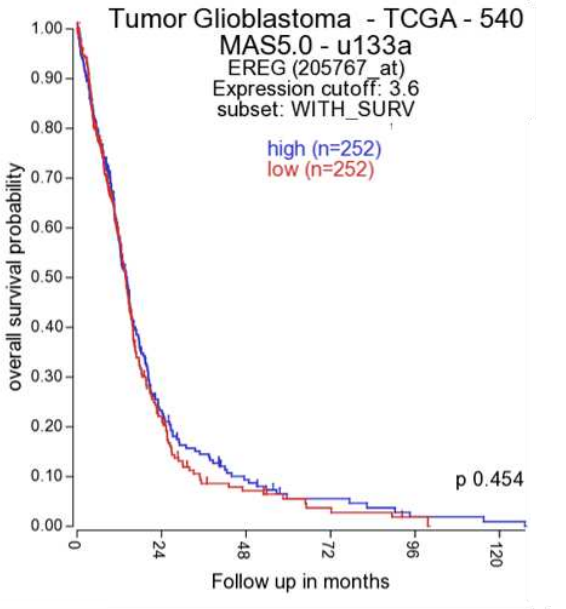

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
HBEGF (203821_at)
Expression cutoff: 112.8
subset: WITH_SURV
high (n=126)
low (n=378)
p 0.048
overall survival probability
Follow up in months

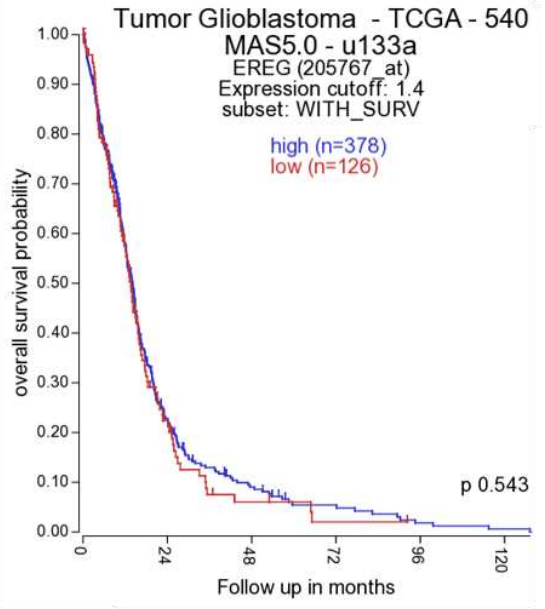

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
TGFA (205016_at)
Expression cutoff: 32.4
subset: WITH_SURV
high (n=252)
low (n=252)
p 0.195
overall survival probability
Follow up in months

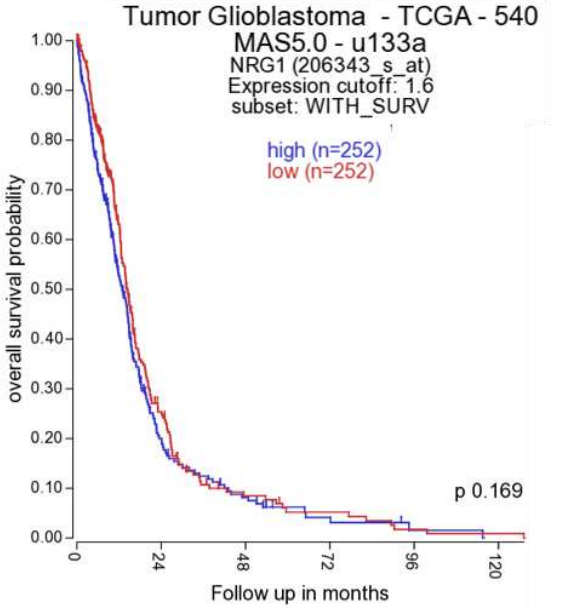

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
TGFA (205016_at)
Expression cutoff: 19.3
subset: WITH_SURV
high (n=378)
low (n=126)
p 0.360
overall survival probability
Follow up in months

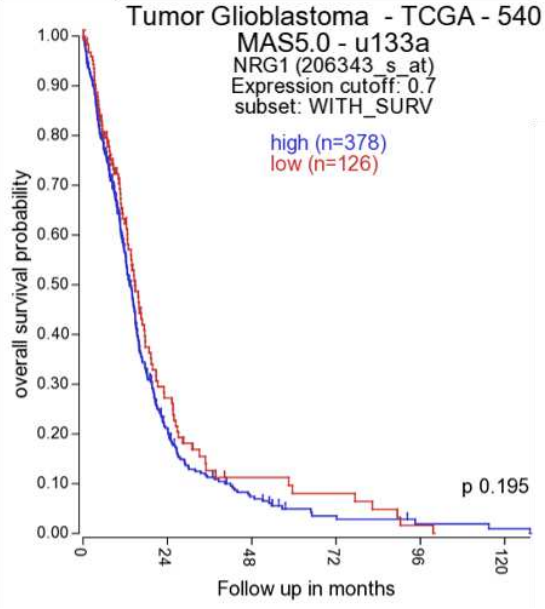

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
BTC (207326_at)
Expression cutoff: 2.1
subset: WITH_SURV
high (n=252)
low (n=252)
p 0.067
overall survival probability
Follow up in months

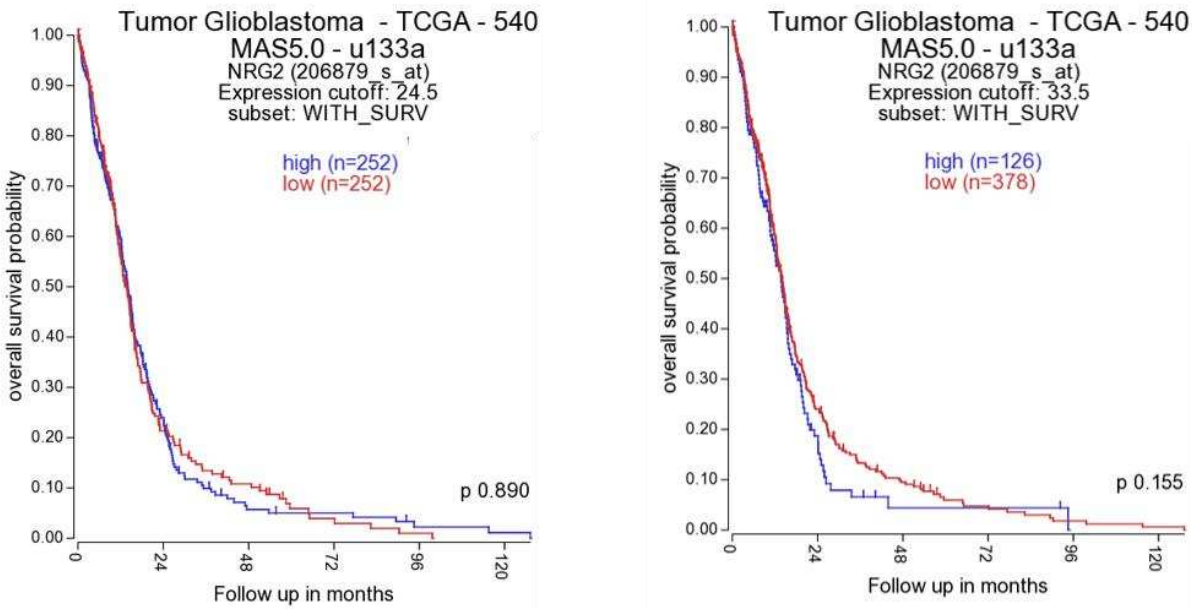

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
BTC (207326_at)
Expression cutoff: 0.7
subset: WITH_SURV
high (n=378)
low (n=126)
p 0.047
overall survival probability
Follow up in months


Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
EREG (205767_at)
Expression cutoff: 3.6
subset: WITH_SURV
high (n=252)
low (n=252)
p 0.454
overall survival probability
Follow up in months

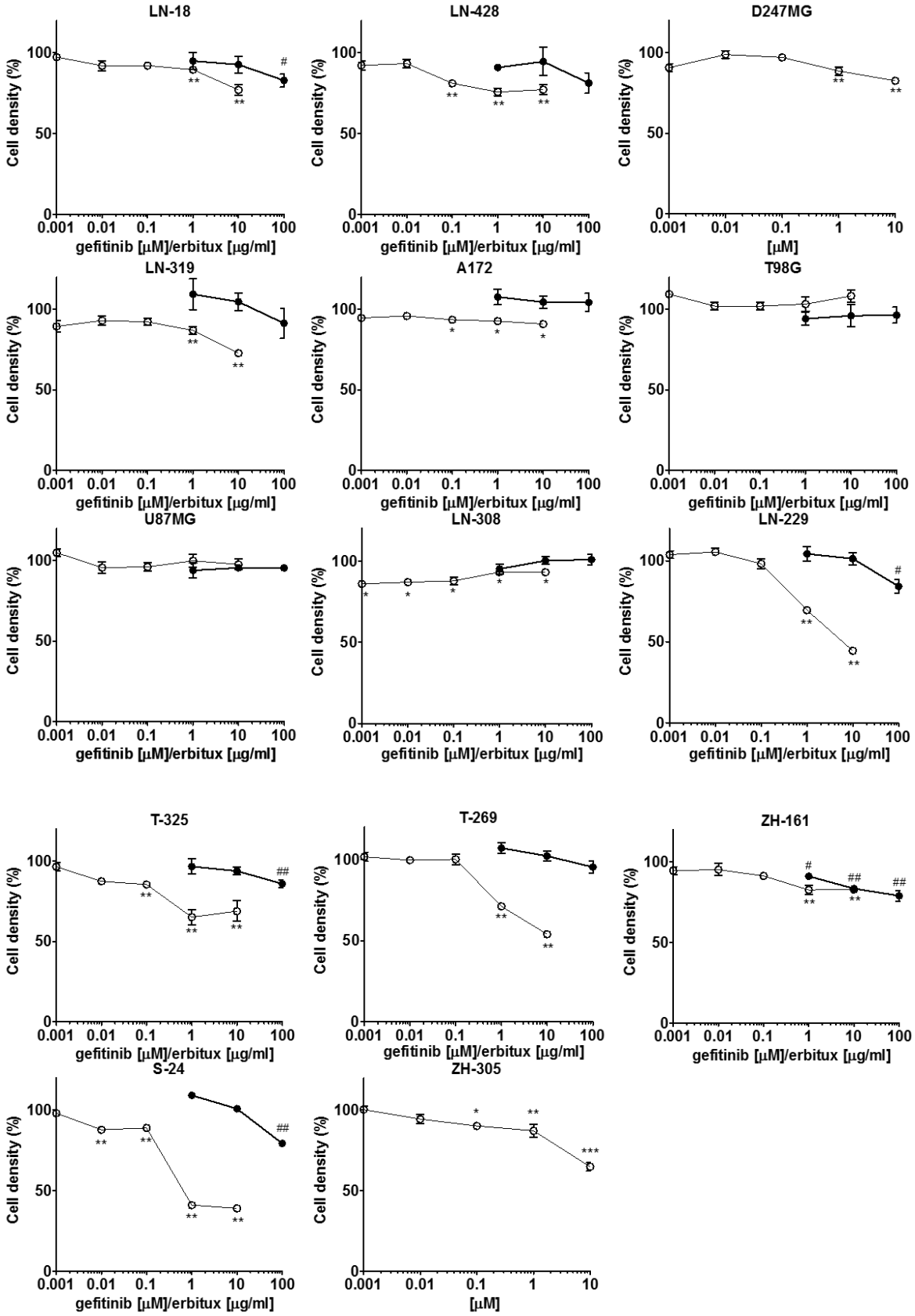

Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
EREG (205767_at)
Expression cutoff: 1.4
subset: WITH_SURV
high (n=378)
low (n=126)
p 0.543
overall survival probability
Follow up in months


Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
NRG1 (206343_s_at)
Expression cutoff: 1.6
subset: WITH_SURV
high (n=252)
low (n=252)
p 0.169
overall survival probability
Follow up in months


Tumor Glioblastoma - TCGA - 540
MAS5.0 - u133a
NRG1 (206343_s_at)
Expression cutoff: 0.7
subset: WITH_SURV
high (n=378)
low (n=126)
p 0.195
overall survival probability
Follow up in months



Supplementary Figure 2. **Association of gene expression levels of *EGF* family ligands with survival in glioblastoma patients.** Kaplan Meier survival curves comparing gene expression status (high or low) shown as in Supplementary Figure S1.


LN-18
LN-428
D247MG
LN-319
A172
T98G
U87MG
LN-308
LN-229
T-325
T-269
ZH-161
S-24
ZH-305
Cell density (%)
gefitinib [μM]/erbitux [μg/ml]
[μM]
0
50
100
0.001
0.01
0.1
1
10
100

Supplementary Figure 3. **Growth inhibitory effects of gefitinib and erbitux in glioma cells *in vitro*.** Metabolic activity reflecting number of viable cells was assessed by MTT assay at 72 h of exposure to gefitinib or erbitux. Data are presented as mean ± SEM normalized to untreated cells in triplicate or sixtuplicate (n=2) (*$p<0.05$, **$p<0.01$, effect of gefitinib compared to control, $^{\#}p<0.05$, $^{\#\#}p<0.01$, effect of erbitux compared to control, one-way ANOVA with Bonferroni correction).

Supplementary Table 1. **Source of glioma-initiating cell (GIC) models: patient and tumor characteristics.**

	Age	Gender	Histological diagnosis
T-325	49	Male	Recurrent glioblastoma
T-269	68	Male	Glioblastoma
S-24	44	Female	Recurrent glioblastoma
ZH-161	57	Male	Recurrent glioblastoma
ZH-305	78	Female	Recurrent glioblastoma

Supplementary Table 2. **Correlation analyses for ERBB family members in glioma cell lines.***

Basal levels (GIC+LTC) receptors	ERBB1 protein	pEGFR (ERBB1) protein	ERBB2 mRNA	ERBB2 protein	ERBB3 mRNA	ERBB3 protein	pERBB3 protein	ERBB4 mRNA
EGFR (ERBB1) mRNA	r=-0.31, p=0.27 r=-0.43, p=0.24 r=0.41, p=0.49	r=0.28, p=0.33 **r=0.70, p=0.03** r=0.08, p=0.90	r=0.43, p=0.12 **r=0.67, p=0.05** r=0.15, p=0.81	r=-0.28, p=0.32 r=-0.4, p=0.28 r=0.39, p=0.50	r=-0.23, p=0.42 r=-0.24, p=0.53 **r=0.88, p=0.05**	r=-0.23, p=0.42 r=-0.32, p=0.41 r=0.75, p=0.15	r=-0.37, p=0.19 r=-0.61, p=0.1 **r=0.91, p=0.03**	r=0.09, p=0.77 r=0.67, p=0.07 r=-0.41, p=0.49
EGFR (ERBB1) protein		r=-0.22, p=0.45 r=-0.50, p=0.17 r=-0.29, p=0.63	r=-0.16, p=0.58 r=-0.17, p=0.65 r=-0.30, p=0.61	r=-0.09, p=0.77 r=0.10, p=0.79 r=0.39, p=0.51	r=-0.003, p=0.99 r=0.004, p=0.99 **r=-0.88, p=0.05**	r=0.01, p=0.98 r=0.01, p=0.97 r=-0.63, p=0.25	r=-0.17, p=0.55 r=-0.15, p=0.68 **r=-0.95, p=0.01**	r=0.43, p=0.14 r=0.26, p=0.54 r=0.52, p=0.37
pEGFR (ERBB1) protein			r=-0.06, p=0.83 **r=0.73, p=0.02** r=-0.38, p=0.52	r=-0.41, p=0.14 r=-0.15, p=0.70 r=-0.35, p=0.55	r=-0.21, p=0.47 r=--0.07 p=0.87 r=0.38, p=0.53	r=-0.23, p=0.43 r=-0.08, p=0.83 r=0.38, p=0.53	r=-0.51, p=0.06 r=-0.37, p=0.32 r=0.20, p=0.75	r=-0.16, p=0.61 r=0.32, p=0.43 **r=-0.88, p=0.05**
ERBB2 mRNA				**r=0.57, p=0.03** r=0.49, p=0.17 r=0.64, p=0.24	r=0.51, p=0.06 r=0.49, p=0.18 r=0.5, p=0.38	r=0.51, p=0.06 r=0.48, p=0.19 r=0.63, p=0.26	r=0.15, p=0.6 r=-0.02, p=0.95 r=0.51, p=0.38	r=0.06, p=0.85 r=0.40, p=0.32 r=0.43, p=0.47
ERBB2 protein					**r=0.9, p=0.0001** **r=0.91, p=0.0006** r=-0.2, p=0.74	**r=0.9, p=0.0001** **r=0.91, p=0.0006** r=-0.15, p=0.81	**r=0.67, p=0.008** r=0.62, p=0.08 r=-0.12, p=0.84	r=-0.15, p=0.60 r=-0.14, p=0.74 r=0.5, p=0.39
ERBB3 mRNA						**r=0.99, p=0.0001** **r=0.99, p=0.0001** r=-0.68, p=0.21	**r=0.56, p=0.03** r=0.54, p=0.13 **r=0.87, p=0.05**	r=-0.08, p=0.78 r=-0.02, p=0.94 r=-0.38, p=0.52
ERBB3 protein							**r=0.58, p=0.03** r=0.56, p=0.12 r=0.62, p=0.26	r=-0.08, p=0.78 r=-0.02, p=0.96 r=0.3, p=0.63
pERBB3 protein								r=-0.28, p=0.35 r=-0.33, p=0.41 r=-0.42, p=0.50

*LTC and GIC pooled (upper lane), or LTC alone (middle lane) or GIC alone (lower lane).

Supplementary Table 3. **Correlation analyses for EGF ligand family members in glioma cell lines.***

Basal levels (GIC+LTC) ligands	NRG1 mRNA	TGF-α mRNA	EREG mRNA	BTC mRNA
EGF mRNA	**r=0.68, p=0.01** **r=0.69, p=0.04** r=0.87, p=0.12	r=-0.18, p=0.54 r=-0.25, p=0.51 r=0.23, p=0.7	r=0.16, p=0.57 r=-0.03, p=0.92 r=0.49, p=0.4	r=0.04, p=0.88 r=-0.43, p=0.25 r=0.46, p=0.43
NRG1 mRNA		r=0.02, p=0.94 r=-0.06, p=0.86 r=0.93, p=0.07	r=-0.15, p=0.60 r=-0.19, p=0.62 **r=0.99, p=0.008**	r=-0.3, p=0.31 **r=-0.62, p=0.05** **r=0.9, p=0.001**
TGF-α mRNA			r=-0.02, p=0.94 r=-0.17, p=0.64 r=0.85, p=0.07	r=0.03, p=0.91 r=-0.03, p=0.92 **r=0.91, p=0.03**
EREG mRNA				**r=0.91, p=0.0001** r=0.33, p=0.39 **r=0.99, p=0.002**

*LTC and GIC pooled (upper lane), or LTC alone (middle lane) or GIC alone (lower lane).

Supplementary Table 4. **Correlation analyses for EGF receptor and ligand family members in glioma cell lines.***

Basal levels (GIC+LTC) ligands	ERBB1 mRNA	ERBB1 protein	pEGFR (ERBB1) protein	ERBB2 mRNA	ERBB2 protein	ERBB3 mRNA	ERBB3 protein	pERBB3 protein	ERBB4 mRNA
EGF mRNA	**r=0.57, p=0.03** r=0.64, p=0.06 r=0.39, p=0.52	r=-0.01,p=0.96 r=0.12, p=0.78 r=-0.35, p=0.58	r=0.1, p=0.76 r=0.46, p=0.22 r=0.42, p=0.47	r=0.39, p=0.15 r=0.36, p=0.33 r=0.61, p=0.28	r=-0.16, p=0.58 r=-0.28, p=0.46 r=0.3, p=0.63	r=-0.17, p=0.56 r=-0.24, p=0.53 r=0.73, p=0.16	r=-0.18, p=0.53 r=-0.25, p=0.51 r=0.31, p=0.61	r=-0.36, p=0.19 r=-0.57, p=0.10 r=0.43, p=0.46	r=0.1, p=0.74 **r=0.7, p=0.05** r=-0.14, p=0.82
NRG1 mRNA	**r=0.57, p=0.04** r=0.58, p=0.1 r=-0.86, p=0.14	r=-0.43, p=0.14 r=-0.41,p=0.27 r=0.47, p=0.53	r=-0.1, p=0.74 **r=0.76, p=0.02** r=0.21, p=0.79	**r=0.75, p=0.003** **r=0.7, p=0.04** r=0.64, p=0.36	r=0.19, p=0.53 r=0.006, p=0.98 r=0.85, p=0.15	r=0.08, p=0.79 r=-0.02, p=0.95 r=0.76, p=0.24	r=0.07, p=0.80 r=-0.03 p=0.93 r=-0.36, p=0.63	r=0.13, p=0.66 r=-0.09, p=0.81 r=0.11, p=0.89	r=-0.07, p=0.82 r=0.46, p=0.24 r=0.06, p=0.94
TGF-α mRNA	r=-0.26, p=0.38 r=-0.2, p=0.49 r=-0.68, p=0.2	r=0.05, p=0.85 r=0.02, p=0.97 r=0.71, p=0.17	r=-0.19,p=0.51 r=-0.07, p=0.86 r=-0.22,p=0.72	r=0.49, p=0.07 r=0.48, p=0.19 r=0.33, p=0.59	**r=0.86, p=0.0001** **r=0.88, p=0.001** **r=0.89, p=0.04**	**r=0.98, p=0.0001** **r=0.99, p=0.0001** r=-0.44, p=0.46	**r=0.98, p=0.0001** **r=-0.99,p=0.0001** r=-0.4, p=0.48	r=0.49, p=0.07 r=0.48, p=0.19 r=-0.5, p=0.38	r=-0.02, p=0.96 r=0.02, p=0.96 r=0.51, p=0.39
EREG mRNA	r=-0.3, p=0.29 r=-0.3, p=0.47 r=-0.47, p=0.42	r=0.08, p=0.78 r=-0.43, p=0.25 r=0.37, p=0.54	r=0.24, p=0.40 r=-0.28, p=0.45 r=0.22, p=0.72	r=-0.2, p=0.47 r=-0.58, p=0.1 r=0.34, p=0.58	r=-0.19, p=0.51 r=-0.32, p=0.3 r=0.82, p=0.09	r=-0.11, p=0.69 r=-0.18, p=0.63 r=-0.12, p=0.85	r=-0.12, p=0.68 r=-0.18, p=0.63 r=-0.48, p=0.41	r=-0.12, p=0.68 r=0.027, p=0.94 r=-0.15, p=0.8	r=0.08, p=0.78 r=-0.17, p=0.68 r=0.02, p=0.97
BTC mRNA	r=-0.42, p=0.13 r=-0.54, p=0.13 r=-0.54, p=0.35	r=0.24, p=0.42 r=-0.03, p=0.94 r=-0.48, p=0.42	r=0.15,p=0.61 r=-0.5, p=0.39 r=0.14, p=0.82	r=-0.20, p=0.48 r=-0.57, p=0.1 r=0.33, p=0.58	r=-0.09., p=0.76 r=-0.07, p=0.85 r=0.84, p=0.07	r=-0.08, p=0.77 r=-0.09, p=0.80 r=-0.18, p=0.76	r=-0.08., p=0.87 r=-0.09, p=0.81 r=-0.48, p=0.41	r=-0.17, p=0.55 r=-0.17, p=0.65 r=-0.26, p=0.66	r=0.12, p=0.68 r=-0.56, p=0.15 r=0.14, p=0.82

*LTC and GIC pooled (upper lane), or LTC alone (middle lane) or GIC alone (lower lane).